splitting of peptide bonds only when the native but not when the heated protein is used as the substrate. In these experiments the alkali uptake by a mixture of 1 mg. chymotrypsin and 10 ml. of a 1% solution of serum albumin was measured at *p*H 7.5.

Thus it would seem that in the presence of salt the denatured albumin can still act as a substrate but not as an inhibitor. The inference would be that the inhibition by serum albumin involves salt sensitive groups that are not essential in its reaction as a substrate. This duality is also suggested by the observation that the Michaelis constants of a variety of substrates (see above) are of the order of 10^3–10^5 times as high as the apparent inhibitor constants (I_{50}-values) of serum albumin and the other proteins of Table II.

Comparison with Other Inhibiting Proteins.—An inhibitory action by protein fractions from serum and other sources on various proteolytic enzymes, including chymotrypsin, has been observed on numerous occasions.[14] However, to the author's knowledge a potent inhibition of the type shown above has never been ascribed to the serum albumin molecule or to other proteins, such as those in Table II. The reason for this could be that salts, applied as buffers or otherwise, were present in concentrations too high to observe this inhibition.

Since it occurs at protein concentrations of the order of 10^{-7} *M*, the inhibition is of the same order of magnitude as that by a typical trypsin and chymotrypsin inhibitor, such as the one that has been obtained from soybean.[15] However, the latter inhibition is of a different type because the effect of soybean inhibitor decreases by heating and is not reversed by salt. An experiment at *p*H 7.5 with 1 mg. of chymotrypsin, 0.1 mg. of soybean inhibitor and 10 ml. of 0.012 *M* MH as the substrate showed an inhibition of 43%, while the heated inhibitor gave less than 10% inhibition. The inhibition was 100% with 1 mg. soybean inhibitor, even in the presence of 0.04 *M* KCl. Thus it would seem that, in contrast to our case, the inhibition by the soybean protein is connected with its secondary or tertiary structure and that ionic forces may not play a predominant role.

(14) For a review with references on this subject see: M. Laskowski and M. Laskowski, Jr., "Adv. in Protein Chemistry," M. L. Anson and K. Bailey, Eds., Vol. IX, Academic Press, Inc., New York, N. Y., 1954, p. 203.

(15) J. H. Northrop, M. Kunitz and R. M. Herriott, "Crystalline Enzymes," Columbia University Press, New York, N. Y., 1948, p. 162.

NOTE ADDED IN PROOF.—In the meantime it has been found that a salt reversible inhibition, similar to that by serum albumin, is also shown by other charged polymers such as carboxymethylcellulose (soluble sodium salt) and nucleic acids. This would give support to the supposition that the inhibition by serum albumin is unrelated to the fact that it is a substrate for the enzyme.

Acknowledgments.—The preliminary work for this investigation was carried out in the Department of Chemistry of the Carlsberg Laboratory in Copenhagen, Denmark. The author gratefully acknowledges the hospitality of the late Professor K. U. Linderstrøm-Lang and the coöperation of the Carlsberg Staff, especially Dr. M. Ottesen and Civilingeniør A. Johansen. This investigation was supported by Grant Nr. C 2289 (C6) from the National Cancer Institute, National Institutes of Health, U. S. Public Health Service.

[CONTRIBUTION FROM THE CHEMICAL LABORATORY OF NORTHWESTERN UNIVERSITY, EVANSTON, ILLINOIS]

"Denaturation" of a Synthetic Polymer and its Relationship to Protein Denaturation

BY IRVING M. KLOTZ AND VIRGINIA H. STRYKER

RECEIVED JANUARY 2, 1960

A conjugate has been prepared having a $(CH_3)_2N$- group attached to a polymer (polyvinylpyrrolidone) which cannot form intramolecular hydrogen bonds. The acidity constants of this polymer conjugate in water and in urea have been compared with those of a protein conjugate. Parallel shifts in pK_a are observed with polymer and protein. Since urea denatures proteins, it must also "denature" the polymer lacking hydrogen bonds. It is suggested, therefore, that the primary step in denaturation is a perturbation of solvent-(macromolecular) solute interactions, rather than a disruption of intramolecular hydrogen bonds.

Introduction

It has been suggested recently[1] that many aspects of the behavior of proteins in solution (*e.g.*, masked groups, reversible denaturation, shifts in pK_a's) might be interpreted advantageously in terms of the crystalline nature of the hydration water of the macromolecule. Analogy was drawn between the stabilizing effect of non-polar molecules (for examples, see Table I) on the lattice structure of water leading to crystalline hydrates,[2-4] and the proposed stabilizing influence of side chains of non-polar amino-acid residues (for examples, see Table I) on the lattice of hydration water of a protein molecule. From this viewpoint, the denaturing effect of urea is considered to involve first a disruption of the hydration lattice, followed, when denaturation is irreversible, by a disruption of the framework of the macromolecule itself.

In this connection an intriguing experiment would be to examine the effect of urea in some macromolecular system which does not have both hydrogen donor and hydrogen acceptor groups to stabilize its structure. In such a system any influence of urea could not be attributed to disruption of intramolecular hydrogen bonds but would have to be ascribed to some other cause.

(1) I. M. Klotz, *Science*, **128**, 815 (1958).

(2) W. F. Claussen and M. F. Polglase, THIS JOURNAL, **74**, 4817 (1952).

(3) M. v. Stackelberg and H. R. Müller, *Z. Elektrochem.*, **58**, 25 (1954).

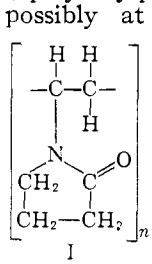
(4) M. v. Stackelberg and B. Meuthen, *ibid.*, **62**, 130 (1958).

TABLE I

COMPARISON OF HYDRATE FORMERS WITH AMINO ACID RESIDUES

Some molecules forming crystalline hydrates	Some amino acid side chains	
CH_4	$-CH_3$	(Ala)
$CH_2(CH_3)_2$	$-CH(CH_3)_2$	(Val)
$CH_3-CH(CH_3)_2$	$-CH_2-CH(CH_3)_2$	(Leu)
CH_3-SH	$-CH_2-SH$	(Cys)
CH_3-S-CH_3	$-CH_2-CH_2-S-CH_3$	(Met)
	$-CH_2-$ 	(Phe)

Two further requirements for the polymer are that it be soluble in water and that it exhibit some interactions similar to a protein. After some consideration, polyvinylpyrrolidone, (I), was chosen. Except possibly at the termini, this



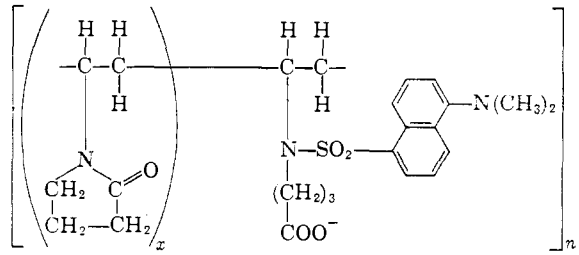
I

polymer contains no hydrogen-donor groups and hence can form no intra- (or inter-) macromolecular hydrogen bonds. It is very soluble in water. Furthermore, like serum albumin it forms complexes with anions and neutral organic molecules,[5-7] although with only about one-third the affinity shown by the protein.[8] In addition, as will be shown below, it shifts the pK_a of a covalently-linked acid-base substituent in the same direction, although not as much, as does serum albumin. Polyvinylpyrrolidone thus seems well-suited as a test macromolecule which we might attempt to "denature" with urea, even though it can form no intramolecular hydrogen bonds.

Experimental

Preparation of Conjugates.—Polyvinylpyrrolidone was obtained from the General Aniline and Film Corporation. The sample had a K number of 30, which according to the manufacturer corresponds to an average molecular weight (determined by osmotic pressure) of about 40,000. To produce a site where a conjugate could be readily attached, polyvinylpyrrolidone was subjected to mild hydrolytic conditions[9] to split just a few of the amide bonds [see (I)] into (secondary) amino and carboxyl groups. The secondary amino group then provided a site for formation of a sulfonamide (II) through reaction of the polymer with 5-dimethylaminonaphthalene-1-sulfonyl chloride.

In a typical preparation, 3.7 g. of polyvinylpyrrolidone was dissolved in 100 ml. of 8 M hydrochloric acid and placed in an oven at about 110° for 45 hr. The slightly hydrolyzed solutions were then dialyzed with frequent changes of distilled water until the pH of the polymer solution rose to 4.9. This solution was then passed through a mixed-bed ion-exchanger [Amberlite MB-1] to remove the last traces of hydrochloric acid. The modified polymer was recovered by lyophilization.



II

The number of rings hydrolyzed was determined by alcoholic-base titration as used for simple amino acids.[10] A blank titration was run with unhydrolyzed polyvinylpyrrolidone. The fraction of pyrrolidone rings hydrolyzed in different samples varied from 1 in 13 to 1 in 38. For the pK experiments described below, the sample of polymer containing (on the average) 1 amino acid per 38 monomeric groups was used.

For conjugation with the sulfonyl chloride, 0.47 g. of hydrolyzed polyvinylpyrrolidone was weighed into 100 ml. of 0.1 M Na_2HPO_4. Twenty five mg. of 5-dimethylaminonaphthalene-1-sulfonyl chloride in 10 ml. of acetone was added and the mixture allowed to stand overnight in the refrigerator. The mixture was then dialyzed for 8 hr. against 0.1 M sodium chloride, so that chloride anions could displace any hydrolyzed sulfonate which might be bound to polymer non-covalently. Thereafter the mixture was dialyzed against distilled water, with frequent changes of water for a period of three days. A small sample of solution was placed on a paper strip and developed with a mixed solvent; formic acid (98–100%), water, secondary butanol = 15,15,70. A brilliant yellow-green fluorescent spot stayed at the origin, but no other fluorescence was observed on the paper. Since the free hydrolyzed sulfonic acid is known[11] to move with an R_f value of 0.29 and to show a blue-green fluorescence, it would seem reasonable to conclude, therefore, that all of the naphthalene dye present was covalently bound to the polymer. When paper chromatography indicated that the polymer conjugate had been freed from non-bound naphthalene sulfonate, the solid conjugate was isolated by lyophilization. The intensity of absorption of a sample was measured in water at 340 mμ. If we assume a molecular extinction coefficient[11] of 3300 l. mole^{-1} cm.$^{-1}$, then an average of about 2 dye molecules were bound on each macromolecule.

Conjugates of glycine and of bovine serum albumin were prepared by procedures described previously.[12]

Titration Procedure.—Polyvinylpyrrolidone-dye conjugate was dissolved in water to give a solution (about 0.3 %) with an optical density at 340 mμ of about 0.7. The pH was then measured and the absorption spectrum obtained over the range of 310–400 mμ. A portion of this solution was then adjusted to a more acid (or more basic) pH and the spectrum obtained again. The procedure was repeated with further samples of solution and different quantities of acid (or base) until enough data had been collected to provide a smooth spectrophotometric titration curve. Suitable corrections were made for dilution of the solution by added acid or base. From the titration curves, pK_a's were evaluated by methods described previously.[12,13]

As a control a similar titration was carried out with partly hydrolyzed polyvinylpyrrolidone containing no coupled dye. The absorption of this solution was constant over the pH range 1 to 7.

Complete duplicate titration curves were obtained in all cases.

Values of pK_a were also obtained from changes of fluorescence with pH. The results are in agreement with those from absorption measurements.

The pH's were measured with a Beckman meter, model G.

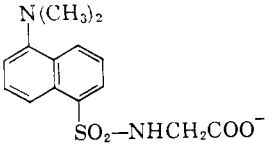
(5) H. Bennhold and R. Schubert, *Z. ges. exptl. Med.*, **113**, 722 (1943).
(6) C. Wunderly, *Arzneimittel-Forsch.*, **4**, I, 29 (1950).
(7) W. Scholtan, *Makromol. Chem.*, **11**, 131 (1953).
(8) I. M. Klotz and J. Ayers, unpublished experiments.
(9) H. P. Frank, *J. Polymer Sci.*, **12**, 565 (1954).
(10) M. Levy in "Methods in Enzymology," Vol. III, edited by S. Colowick and N. Kaplan, Academic Press, Inc., New York, N. Y., 1956, p. 454.
(11) B. S. Hartley and V. Massey, *Biochim. Biophys. Acta*, **21**, 58 (1956).
(12) I. M. Klotz and H. A. Fiess, *ibid.*, **38**, 57 (1960).
(13) I. M. Klotz and J. Ayers, THIS JOURNAL, **70**, 4078 (1957).

Optical Measurements.—The absorption of light was measured in a Beckman spectrophotometer, model DU, at 25°.

Materials.—Bovine serum albumin was a crystallized sample from Armour and Co. Polyvinylpyrrolidone is described above. The 5-dimethylaminonaphthalene-1-sulfonyl chloride was a purified grade purchased from the California Corporation for Biochemical Research. Acetone was Eastman Kodak spectroscopic grade. Acids and bases were reagent grade. Urea was of reagent grade but was purified further by the procedure recommended by Benesch, Lardy and Benesch.[14] Glycine was C. P. grade of H. M. Chemical Co.

Results and Discussion

Behavior in Water.—As a measure of the intrinsic pK_a of the dimethylanilinium group in the naphthalene dye, the acid–base equilibrium of a conjugate of glycine (III) was studied first. In

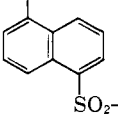


III

water the $-\overset{+}{N}H(CH_3)_2$ group of this molecule shows a pK_a of 3.99 (Table II).

TABLE II

ACIDITY CONSTANTS OF $\overset{+}{H}N(CH_3)_2$ AT 25°



	pK_a for R =		
Solution	Glycine	Bovine serum albumin	Polyvinyl-pyrrolidone
Water	3.99[a]	1.67	2.54
8 *M* Urea	4.18	3.27	3.24

[a] Glycine-dye conjugate dissolved in 1% aqueous polyvinylpyrrolidone had a pK_a of 4.02.

The same aromatic molecule attached to bovine serum albumin, however, has a pK_a of 1.67, a shift of over 2.3 *p*H units. The conjugate of polyvinylpyrrolidone in turn has a pK_a of 2.54, a shift of almost 1.5 *p*H units from that of (III).

Thus we find that "masking" occurs when an ionizing group is attached to a synthetic polymer containing virtually no hydrogen bonds between monomer residues, as well as in a hydrogen-bonded protein. In the conjugate of the polymer it seems very far-fetched to invoke an electrostatic cause to account for the downward shift in pK_a, for on the average only one in each fifty residues can carry a charge. The specific residue to which the dimethylaminonaphthalene is attached [see II] does indeed have a carboxylate ion, but this is too far away from the $(CH_3)_2N$- group to have a significant electrostatic influence. In this connection it is pertinent to recall that even in the amino acid series, where the charged groups are much closer, displacement of the $-NH_3^+$ from the α carbon to merely the γ carbon is accompanied by a drop in ΔpK,[15] due to the electrostatic effect of the carboxyl group, from 2.0 to 0.7 *p*H units. In the polymer conjugate the $-\overset{+}{N}H(CH_3)_2$ group is much farther away from the carboxyl group. Furthermore, long before a *p*H as acid as 2.5 has been reached, the COO^- group should be almost completely in the uncharged COOH state and should exert no electrostatic influence at all.[16]

On the other hand, the shift in pK_a of the polyvinylpyrrolidone conjugate can be understood if we assume that this polymer, because of its large content of non-polar groups, is capable of establishing an ice-like hydration sheath similar to that postulated for proteins.[1,13] Such an environment would be partial to the $(CH_3)_2N$ rather than to the charged, water-structure-breaking $(CH_3)_2NH^+$ group. It is this required disorientation of the hydration lattice which may be considered the obstruction to the formation of the acid-form of the amine.

"Denaturation."—Turning to the effect of urea on pK_a, we find that the protein and synthetic polymer behave in a very similar fashion. In both cases the urea "unmasks" the $-N(CH_3)_2$ group and shifts the pK_a back up toward that of the reference compound. In fact, the actual pK_a attained by each macromolecular conjugate, protein or polymer, in urea is the same within experimental error.

This "unmasking" of the $(CH_3)_2N-$ group attached to serum albumin by the addition of urea to the solution is a typical example of the class of phenomena in proteins called "denaturation." Since urea produces the same unmasking effect on the $(CH_3)_2N-$ group attached to polyvinylpyrrolidone, we ought also to call this perturbation of behavior of the synthetic polymer, "denaturation."

In contrast to the protein, however, polyvinylpyrrolidone can hardly form an intramolecularly hydrogen-bonded structure, such as a helix. The original polymer has no hydrogen donor groups, except perhaps at the chain termini. Hydrolyzed polyvinylpyrrolidone has a few side chains (on the average, 1 in 38 residues before the conjugate has been prepared) per macromolecule with hydrogen donor groups. The very small number and geometric position of these makes it very unlikely that they could stabilize any special ordered configuration of the macromolecule. Nevertheless, the effect of urea on the pK_a is comparable to its effect on the pK_a of bovine albumin in which every residue is capable of engaging in intramolecular hydrogen bonding.

We are obliged, therefore, to consider some alternative description of the molecular basis of the denaturing effect of urea. Although urea cannot disrupt hydrogen bonds in polyvinylpyrrolidone because there are (essentially) none, it can presumably still break any hydrogen bonds of water. If we accept, therefore, the views mentioned above

(14) R. E. Benesch, H. A. Lardy and R. Benesch, *J. Biol. Chem.*, **216**, 663 (1955).

(15) A. Neuberger, *Proc. Roy. Soc. (London)*, **A158**, 68 (1937).

(16) One might argue that the COOH group [see (II)] could form a hydrogen bond with the $-N(CH_3)_2$ and thereby lower the pK_a of $\overset{+}{H}N(CH_3)_2$. It is difficult to believe that such a hydrogen bond would form with a distant aromatically-linked nitrogen when an energetically and sterically more favorable oxygen (of $-SO_2-$) is available. Furthermore, such a hydrogen bond ought to perturb the resonance between nitrogen and aromatic ring and hence the spectrum. No shift in peak is observed as the pK is lowered from 6 to 4.5.

that the masking originally observed when the polymer is in water is due to an ice-like hydration lattice, then it follows naturally that the hydrogen-bond breaking power of urea[17] would manifest itself by disrupting the hydration lattice of the polymer. Such an action would account immediately for the upward shift in pK_a of the polyvinylpyrrolidone conjugate.

Since denaturation of this synthetic polymer cannot involve any disruption of intramolecular hydrogen bonds, the question arises whether it is necessary or valid to assume that denaturation of protein molecules always involves a disruption of intramolecular hydrogen bonds. The primary step with protein too might be instead a perturbation of solvent-(macromolecular) solute interactions.

Acknowledgment.—This investigation was assisted by a grant from the National Science Foundation.

(17) It has been suggested by W. Kauzmann [*Adv. Protein Chem.*, **14**, 1 (1959)] that urea disrupts hydrophobic bonds. Such a postulate, however, represents a substantial departure from the generally-accepted view that the action of urea depends on its ability to break hydrogen bonds. Hydrophobic bonds, as usually pictured, are not based on direct hydrogen-bonding. Urea does indeed form inclusion complexes with hydrocarbons, as mentioned by Kauzmann, but only if they are straight-chain aliphatic molecules (not branched not aromatic) with a length of six or more carbon atoms [E. Bengen and W. Schlenk, Jr., *Experientia*, **5**, 200 (1949)]; hydrophobic amino acid side chains do not fulfill these requirements.

[CONTRIBUTION FROM THE DEPARTMENT OF CHEMISTRY, FACULTY OF SCIENCE AND THE RESEARCH INSTITUTE FOR CATALYSIS, HOKKAIDO UNIVERSITY, SAPPORO, JAPAN]

Kinetic Analysis of the Pyrophosphate–Myosin B System by the Use of the Light-scattering Method

BY FUMI MORITA AND YUJI TONOMURA

RECEIVED DECEMBER 24, 1959

For the explanation of the change produced by pyrophosphate (PP) in the shape of myosin B (M) in 0.6 M KCl solution, the following mechanism was postulated; $M + PP \underset{k_{-1}}{\overset{k_1}{\rightleftarrows}} MPP$ (1), $MPP \underset{k_{-2}}{\overset{k_2}{\rightleftarrows}} M^*PP$ (2), where the asterisk indicates the changed state of myosin B. All the experimental results obtained by the use of the light-scattering method were in accord with this mechanism. The dissociation constant of the over-all reaction K and the forward rate constant of step 2 k_2, were determined from the decrements at equilibrium and at the transient states, respectively, in the light scattered by myosin B caused by PP. In the presence of Ca^{++} in concentrations higher than 0.3 mM, the dissociation constant of step 1 K_1 could be determined according to the transient method and that of step 2 K_2 could be calculated from the values of K and K_1. The mean values of K_1 and K_2 of the seven preparations tested were 1.1×10^{-3} and 5.2×10^{-2} M, respectively, in the presence of Ca^{++} and at pH 7.9, $D = 82.56$ and at 5°. The steady-state velocity for the reverse direction was measured by the addition of inorganic pyrophosphatase at the equilibrium of the reaction between myosin B and PP. The ratio of the velocity of the reverse reaction to that of the forward one agreed well with the dissociation constant. The enthalpy change (ΔH) of each step was calculated according to the Arrhenius equation; the mean values of ΔH of the steps 1 and 2 are −9.2 and −11 kcal./mole, respectively. The change in electrostatic free energy could be calculated from the dependence of K_1 and K_2 on the dielectric constant of the medium. The values of 11 and −44 kcal./mole were obtained for the steps 1 and 2, respectively. The entropy changes of the steps 1 and 2 were evaluated from the changes in free energy and enthalpy, and the values of −19 and −33 cal./mole deg. have been obtained, respectively. K_2 was nearly independent of pH, while K_1 decreased remarkably at pH above 8. The molecular mechanism of the change in the shape of myosin B was discussed on the basis of the results obtained.

Introduction

The molecular mechanism of the change in the size and shape of myosin B caused by adenosinetriphosphate (ATP) or pyrophosphate (PP) has not been clear until recently, in spite of much effort made by several workers.[1-3] However, it was shown by the use of the light-scattering method and the ultracentrifugal analysis[4,5] that the main components of myosin B become elongated by the addition of ATP or PP. The results obtained by the equilibrium dialysis and the light-scattering method indicated that the myosin B molecule contains one mole of the binding site for PP per 5.6×10^5 g. and that the intensity of the light scattered decreases with an almost constant value, every time one PP molecule is bound to one site of myosin B, each site being equal in its intrinsic affinity for PP.[6]

On the basis of these results the molecular kinetic mechanism for the interaction between myosin B and PP were investigated by the use of the light-scattering method. One of the present authors[7] previously studied the transient light-scattering change of myosin B following the addition of ATP. However, the change in light-scattering used to take place so fast that the present technique of mixing seemed to be somewhat unsatisfactory and the correction on account of ATP hydrolysis was required. Myosin B does not hydrolyze PP. Change in the light scattered proceeds much slower after the addition of PP than after the addition ATP. Therefore, by the use of the transient light-scattering method, the effect on the shape of myosin B can be investigated more quantitatively in the case of PP than in the case of ATP.

(1) J. J. Blum and M. F. Morales, *Arch. Biochem. Biophys.*, **43**, 208 (1953).

(2) A. Weber, *Biochim. Biophys. Acta*, **19**, 345 (1956).

(3) J. Gergely, *J. Biol. Chem.*, **220**, 917 (1956); J. Gergely and H. Kohler, "Conference on the Chemistry of Muscular Contraction," Igakushoin, Tokyo, 1957, p. 14.

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(5) T. Nihei and Y. Tonomura, *J. Biochem.*, **46**, 1355 (1959).

(6) Y. Tonomura and F. Morita, *ibid.*, **46**, 1367 (1959).

(7) Y. Tonomura, S. Watanabe and K. Yagi, *ibid.*, **40**, 27 (1952).